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# Utilization of *Aspergillus niger* Phytase Preparation for Hydrolysis of Phytate in Foods

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## 1. Introduction

The majority of phosphorous in plant seed, especially cereal grains and legumes, is myo-inositol hexaphosphate (phytate or $IP_6$) (Rosa et al., 1999). Structure of $IP_6$ is shown in Figure 1. Figure 2 shows distribution of $IP_6$ in some plant foods. Unrefined cereals and soybean products contain high levels of $IP_6$. Rice bran contains $IP_6$ more than 6,000 mg/100 g of dry matter. $IP_6$ has a strong capability to chelate multivalent metal ions, particularly zinc, calcium, and iron ions, which results in the formation of highly insoluble salts (Nolan et al., 1987; Hotz et al., 2001). The $IP_6$ can be hydrolyzed by enzyme (phytase) and converted to lower myo-inositol phosphates; from inositol pentaphosphate ($IP_5$) to inositol monophosphate ($IP_1$) and myo-inositol. It has been demonstrated that the metal complexes with $IP_1$−$IP_4$ are more soluble than those with $IP_5$ and $IP_6$ (Sandberg et al., 1989). Animal experiments have demonstrated that the ingestion of the meal that contains more than 1% $IP_6$ decreases intestinal absorption of metal ions and induces the metal ion deficiency (Hirabayashi et al., 1998; Grases et al., 2001). In some communities, unrefined cereals are still main ingredients for the diet. In addition, the unrefined grains of rice, wheat, rye, etc., are richer in various minerals, dietary fiber, vitamins, and other bioactive components than refined ones. Therefore, these unrefined cereals are used as food ingredients for bread,

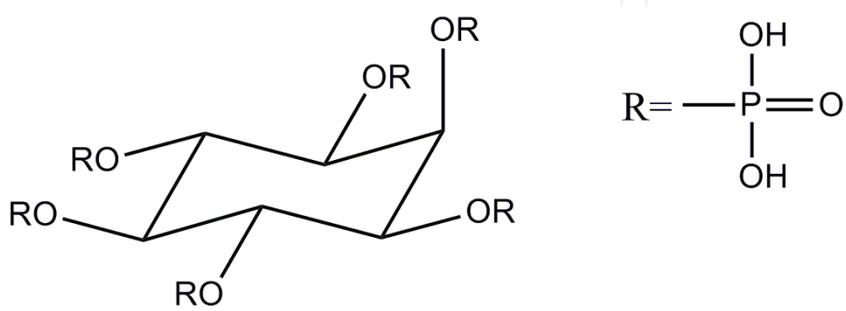
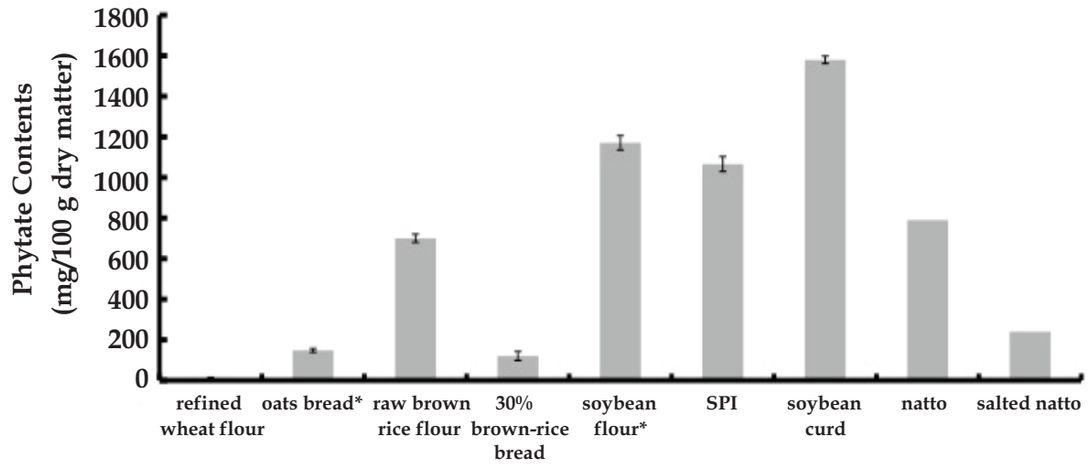



Fig. 1. Structure of phytic acid ($IP_6$)

breakfast cereals and so on due to their high health-promoting activities (Fukui et al., 1997; Haros et al., 2001; Lopez et al., 2001; Porres et al., 2001). In addition to the unrefined cereals, legumes, especially soybean have long history as food ingredient. Recently, soy flour, soy protein isolate, its protease digest, etc. have been formulated and used as protein source for infant formulae, sports drinks, enteral nutrients and also diets for animal, poultry, and fish. However, as mentioned above, these products are also rich in $IP_6$. Then, nutritional disturbances in the absorption of iron and zinc may occur by ingestion of $IP_6$–rich foods (Shaw et al., 1995; Sandberg et al., 1996; Minihane et al., 2002).




* Data from Rosa et al. 1999. Natto; steamed soybean fermented with *Baccillus subtillis*. Salted natto; steamed soybean fermented with *Aspergillus oryzae* and further fermented with wild yeast and lactic acid bacteria in the presence of salt (more than 5%).

Fig. 2. Phytate contents of some cereals and soybean products

It has been demonstrated that the $IP_6$ in foods can be degraded by phytase, which would improve mineral absorption in humans (Sandberg et al., 1996; 2002). On the basis of these facts, some phytase preparations have been formulated and used to reduce $IP_6$ and $IP_5$ levels of some plant-based foods (Haros et al., 2001; Saito et al., 2001; Porres et al., 2001: Matsuo et al., 2005; 2010) and also animal and fish diets (Pallauf & Rimbach, 1997; Sajjadi & Carter, 2003).

In this chapter, the recent application of fungal phytase preparation as food additive and its problem are reviewed. In addition, recent studies for control of the contamination of other enzymes in the phytase preparation are focused.

## 2. Source of phytase

It has been demonstrated that fermentation using lactic acid bacteria and fungus can reduce $IP_6$ levels in soybean flour and bran-enriched bread (Hirabayashi et al., 1995; Harose et al., 2001; Andlid et al., 2004; Leenhardt et al., 2005; Matsuo et al., 2005; Reale et al., 2004; 2007; Palacious et al., 2007; Li et al., 2008; Jorquera et al., 2008; Sanz-Penella et al., 2009). These facts indicate microorganisms involved in the fermentation process can produce phytase.

Indeed, some bacteria, yeasts, and fungi have been demonstrated to produce phytases. As mentioned above, the enzymatic hydrolysis of the $IP_6$ in unrefined cereals and legumes improves mineral absorption in humans (Sandberg et al., 1996; 2002). These facts can lead an idea that microbial phytase can be used as food additive to reduce $IP_6$ level in the plant-based foods. So far to now, phytase preparations have been prepared from *Asperigillus niger*,

recombinant *Aspergillus oryzae*, *Pichia pastoris* etc. in an industrial scale and used as food additive (Greiner & Konietzny, 2006). However, these microbial also produce protease, amylase and etc. As demonstrated in the following sections, a food additive-grade *Asperigillus niger* phytase preparation has significant protease and amylase activities (Matsuo et al., 2010). In some cases, occurrences of protease and amylase in the food additive-grade phytase preparation exerts adverse effects on texture and appearance of the final products.

In the following sections, application of a food additive-grade *Aspergillus niger* phytase preparation on some plant-based foods are introduced. Effect of the phytase treatment not only on $IP_6$ content but also on the other properties will be discussed.

## 3. Application for improvement of nutritional value of animal, poultry, and fish diets

Plant seeds and grains have been used for animal, poultry, and fish diets. As shown in Figure 2, the unfermented soy and cereal products contain high levels of $IP_6$. The occurrence of $IP_6$ in diet may interfere mineral absorption (Sandberg, 2002). In some cases, it may induce serious nutritional problem. Indeed, feeding $IP_6$-containing soy protein isolate retarded growth of fish larvae. To solve these problems, *Aspergillus niger* and *Pichia pastoris* phytase preparation was used to decrease $IP_6$ in the plant-based diet. This treatment significantly improve feeding efficacy. Now, formulated *Aspergillus niger* and *Pichia pastoris* phytases for improvement of feeding efficacy for fish, chicken, animals are commercially available (Pallauf & Rimbach, 1997; Sajjadi & Carter, 2003).

## 4. Application of phytase to isolate conglycinin and glycinin from soy protein isolate

Phytase treatment of soy protein isolate can be carried out in aqueous solution. During the *Asperigillus niger* phytase treatment, protein precipitation occurred (Saito et al., 2001). As well known, major soluble proteins in soybean are glycinin and β-conglycinin, which are classified as globulin. Both proteins are precipitated in acidic condition at approximately pH 4.5. However, predicted isoelectric point of glycinin based on the protein sequence is neutral pH. The glycinin forms complex with $IP_6$ and then it has apparent acidic isoelectric point, which makes glycinin precipitate in acidic condition. The phytase treatment degrades the glycinin-bound $IP_6$ and shifts isoelectric point to neutral pH. Then selective precipitation of glycinin occurs at neutral pH after the phytase treatment of soy protein isolate. This technique is successfully applied to sepration of glycinin and β-conglycinin for food ingredient. It has been demonstrated that β-conglycinin can reduce blood neutral lipid level of animal and human by ingestion (Fukui et al., 2004; Moriyama et al., 2004; Kohno et al., 2006). Now, food-grade β-conglycinin fraction is prepared on the basis of phytase treatment

and following selective precipitation technique. The β-conglycinin fraction is approved to present health claim on lipid metabolism in "Food for the Specific Health Use" in Japan.

## 5. Application for unrefined cereal flour-containing bread making.

Whole grains of wheat, rice, rye, etc., are richer than refined grains in various minerals, dietary fiber, and vitamins. Besides minerals, vitamins, and dietary fiber, the unrefined grains are rich in bioactive components that have health-promoting activities; for example, γ-aminobutyric acid (GABA), γ-oryzanol, polyphenols, and feruric acid. These components show antihypertensive (GABA), antioxidant, and anti-hypercholesterolemic (γ-oryzanol and feruric acid) activities (Sugano et al., 1997; Xu et al., 2001; Hayakawa et al., 2002). However, the consumption of the unrefined whole grains as the steamed and fried forms, are not very prevalent due to the poor swelling and textural properties of the unrefined whole grains. Then, the unrefined whole grains are usually processed to flour and used as bread ingredient, which has been gaining popularity worldwide (Fukui et al., 1997; Haros et al., 2001; Lopez et al., 2001; Porres et al., 2001). As shown in Figure 3, $IP_6$ in the dough mix containing 30% brown rice flour decreases during processing by the action of yeast phytase. However, the relatively higher levels of $IP_6$ remains in the bread containing brown rice flour. As shown in Figure 3, addition of food additive-grade *Aspergillus niger* phytase preparation significantly decreased $IP_6$ level of brown rice-added bread. *Aspergillus niger* phytase has

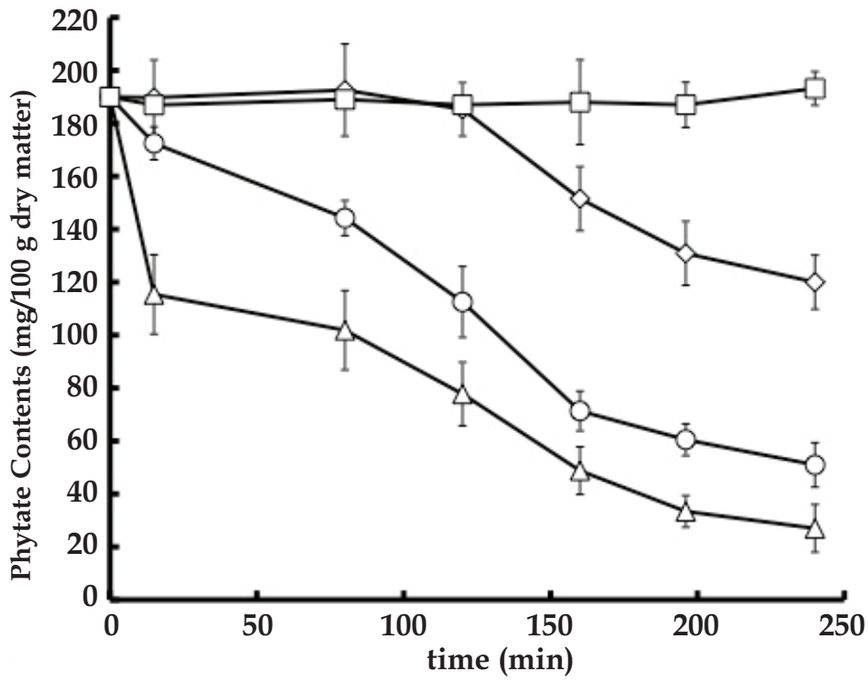



Fig. 3. Changes in phytate contents during bread making process. 0-195 min; mixing and fermentation, 240-min; baking. (□); 30% brown rice flour-containing dough without addition of yeast and phytase, (◊); added with yeast only, (○); added with yeast and 600 U of the *Asparagillus niger* phytase preparation, (△); added with yeast and 3000 U of the phytase preparation. Values are means ±SD, n=3. From Matsuo et al., 2005

been also used to decrease $IP_6$ level in the whole wheat fours (Haros et al., 2001). At early attempt, the flour was pretreated with phytase in water suspension system. It has been, however, demonstrated that direct addition of the phytase preparation into dough mix can reduce $IP_6$ content, which is the easier approach to decrease $IP_6$ level in the unrefined grain flour-containing bread (Haros et al., 2001; Matsuo et al., 2005; 2010). However, addition of high dose of the phytase preparation (3000 U) induced collapse of whole bread crust and deteriorates texture and taste of the final product as shown in Figure 4 E (Matsuo et al., 2005).

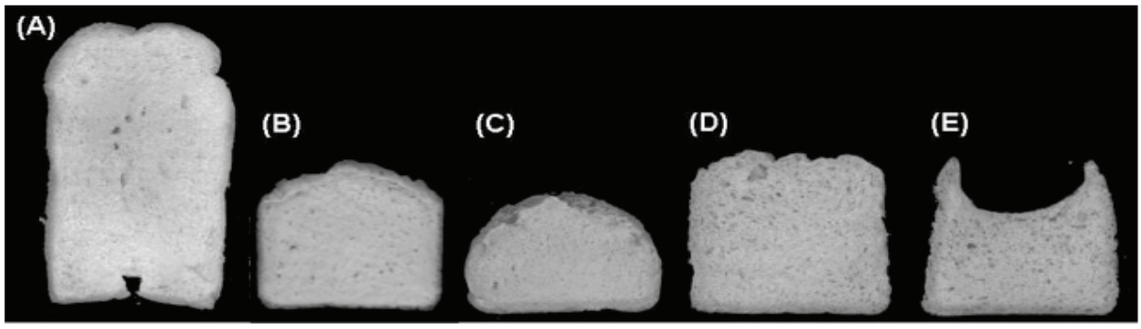



Fig. 4. Effect of addition of brown rice flour and phytase on the loaf volume. (A); wheat bread, (B); 30% brown rice flour bread, (C); 50% brown rice flour bread, (D); 30% brown rice bread flour bread added with 600 U of the phytase preparation; (E) 30% brown rice flour bread added with 3000 U of the phytase preparation. From Matsuo et al., 2005

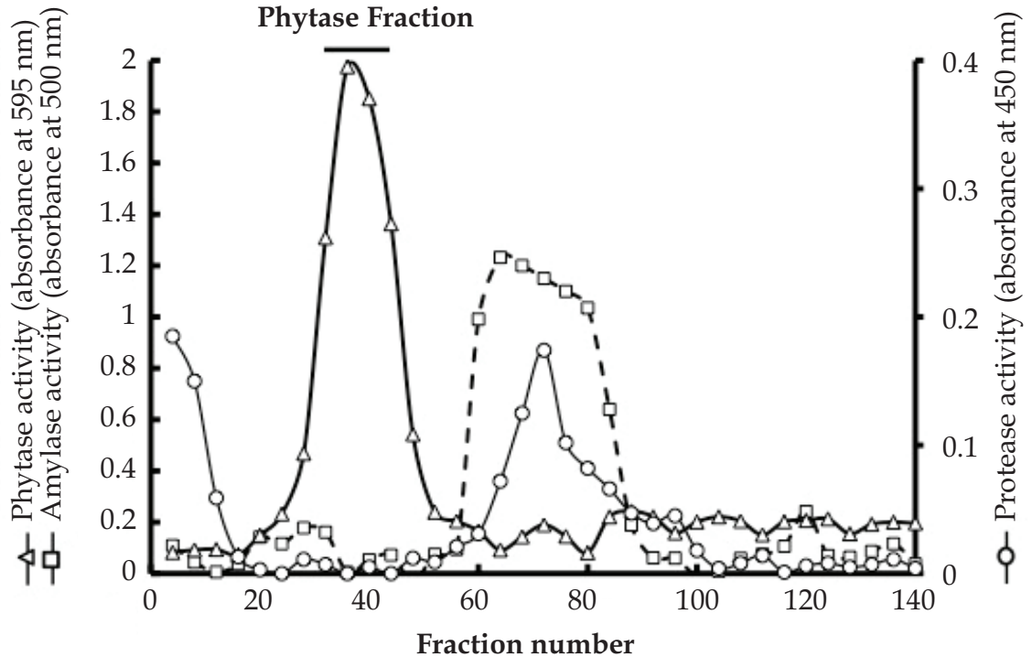



Fig. 5. Separation of phytase from amylase and protease in the crude phytase preparation by anion-exchange chromatography. Fraction indicated by the bar was collected and used as the purified phytase preparation

Two grams of the crude phytase preparation was dissolved in 50 mL of 0.02 M sodium acetate buffer, pH 6.0 and loaded on a column (15 cm × 10 mm i.d.) packed with TSK gel Super Q-Toyopearl 650S (Tosoh Co., Tokyo, Japan) that had been pre-equilibrated with the same buffer. The absorbed proteins were eluted using 200 mL of a linear gradient of 0-0.5 M NaCl in the same buffer at 1.5 mL/min. Fractions were collected every 1 min. From Matsuo et al., 2010.

Figure 5 shows that the food additive-grade phytase preparation contains significant activities of protease and amylase, which can be separated from the phytase activity by anion-exchange column chromatography (Matsuo et al., 2010). Addition of the purified phytase fraction free from protease and amylase activities into the dough mix decreased $IP_6$ level without affecting bread volume (Figure 6). These facts imply that amylase and/or protease are responsible for the collapse of the bread crust by addition of high dose of the commercial phytase preparation. However, it has been difficult to isolate phytase, protease, and amylase activities in enough amounts for the additive purpose by conventional chromatography technique due to high cost of preparative LC system. Then it is difficult to control protease and amylase activities in the phytase preparation, which has limited the use of the fungal phytase preparation as food additive. To solve this problem, a large-scale isolation method for food additive-grade enzyme is necessary. This method should be inexpensive, biocompatible, easy to scale-up. The following section introduces recent advance in purification of phytase by new technique.

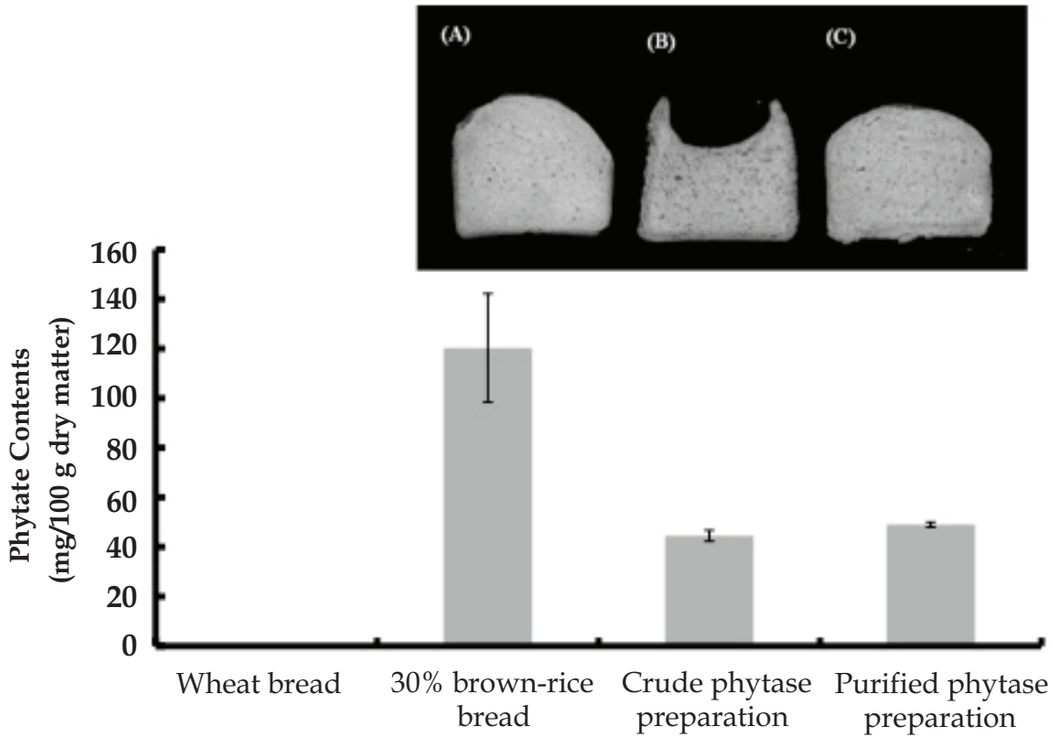



Fig. 6. Effect of crude and purified phytase (3,000 U) on phytate contents and swelling property of 30% brown rice bread. A; 30% brown rice bread, B; added with the crude phytase, C; added with the purified phytase. From Matsuo et al., 2010

## 6. Control of protease and amylase activities in phytase preparation by autofocusing

As mentioned in the previous section, it has been demanded to develop a method for large-scale fractionation of enzymes in crude extract and fermentation broth in order to control contamination of other enzymes in phytase preparation. For fractionation of peptides in the crude digests of food proteins, a preparative ampholyte-free isoelectric focusing has been developed (Hashimoto et al., 2005), which has a potential for purification of enzymes. This technique depends on amphoteric nature of the sample compounds. The crude enzymatic digest of food protein, crude extract, fermentation broth, etc. contain numerous compounds with different isoelectric points. Then these compounds can act as ampholine for isoelectric focusing. This phenomenon is referred to autofocusing (Yata et al., 1996; Akahosi et al., 2000; Hashimoto et al., 2005). This technique does not require harmful reagents and solvents just requires water and agarose gel in sample compartments and diluted phosphoric acid and sodium hydroxide in the electrode compartments. All of them can be used for food processing. Assembly of the apparatus is illustrated in Figure 7. A plastic plate with window is covered with nylon screen and the screen is wetted with hot agarose solution. After standing for few minutes, thin agarose gel layer is formed on the screen. The plates with agarose gel layer are inserted into the slots of the tank as shown in Figure 7. Then the tank can be separated into 12 compartments. Both ends of the compartments are used as electrode compartments. Others are used as sample compartments (10 compartments).

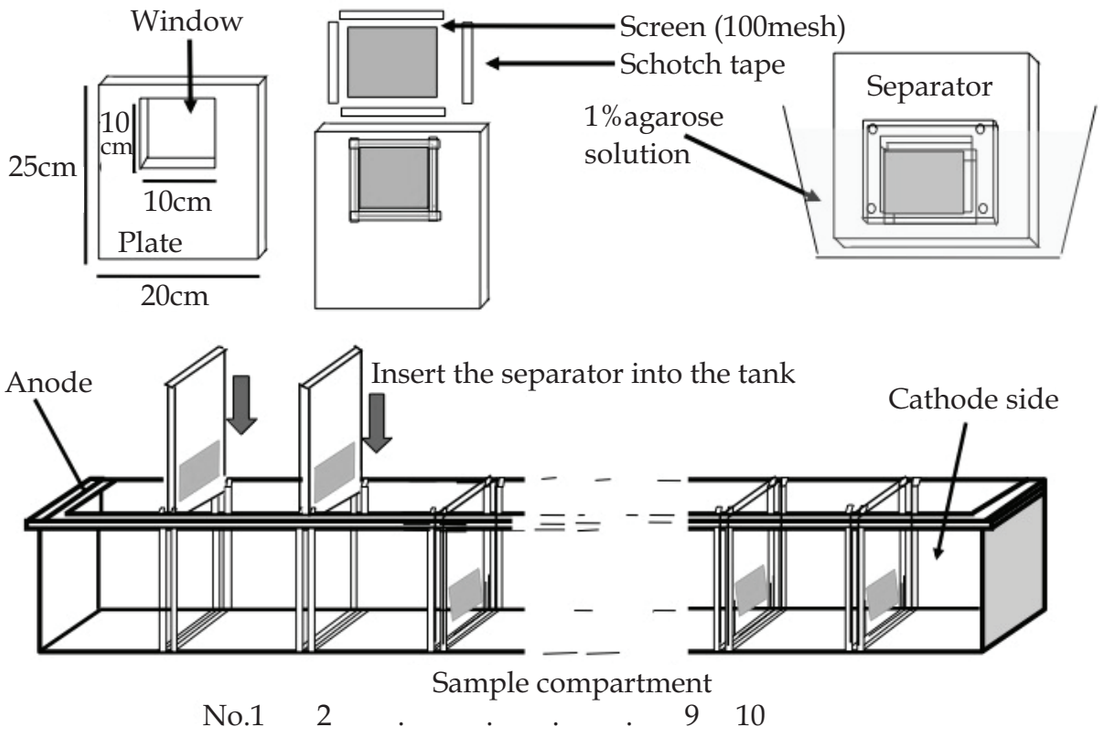



Fig. 7. Schematic drawing for assembly of Autofocusing apparatus. The tank is divided into 12 compartments separated with thin agarose gel layer. Volume of compartment can be changed from 100 mL to 5 L. From Hashimoto et al., 2005

Water-solution of sample is applied into the sample compartments. 0.1 N phosphoric acid and sodium hydroxide are loaded into cathode and anode, respectively. By loading direct electric current into the electrode compartments at constant voltage mode at 500 – 1000 V, the compounds in sample start to migrate to their own isoelectric points. This technique has been successfully used for peptide fractionation (Hashimoto et al., 2005; Higaki-Sato et al., 2006; Park et al., 2008; Murota et al., 2010; Elbarbary et al., 2010).

The food additive-grade phtase preparation also contain many compounds. Then autofocusing was used to purify phytase in the commercial preparation. As shown in Figure 8, pH gradient approximately from 2 to 11 was formed by autofocusing of food additive-grade *Aspergillus niger* phytase preparation. The protease activity was migrated to acidic fractions (Fr. 1-6). The amylase and phytase activities were widely distributed. Fr. 7 has the

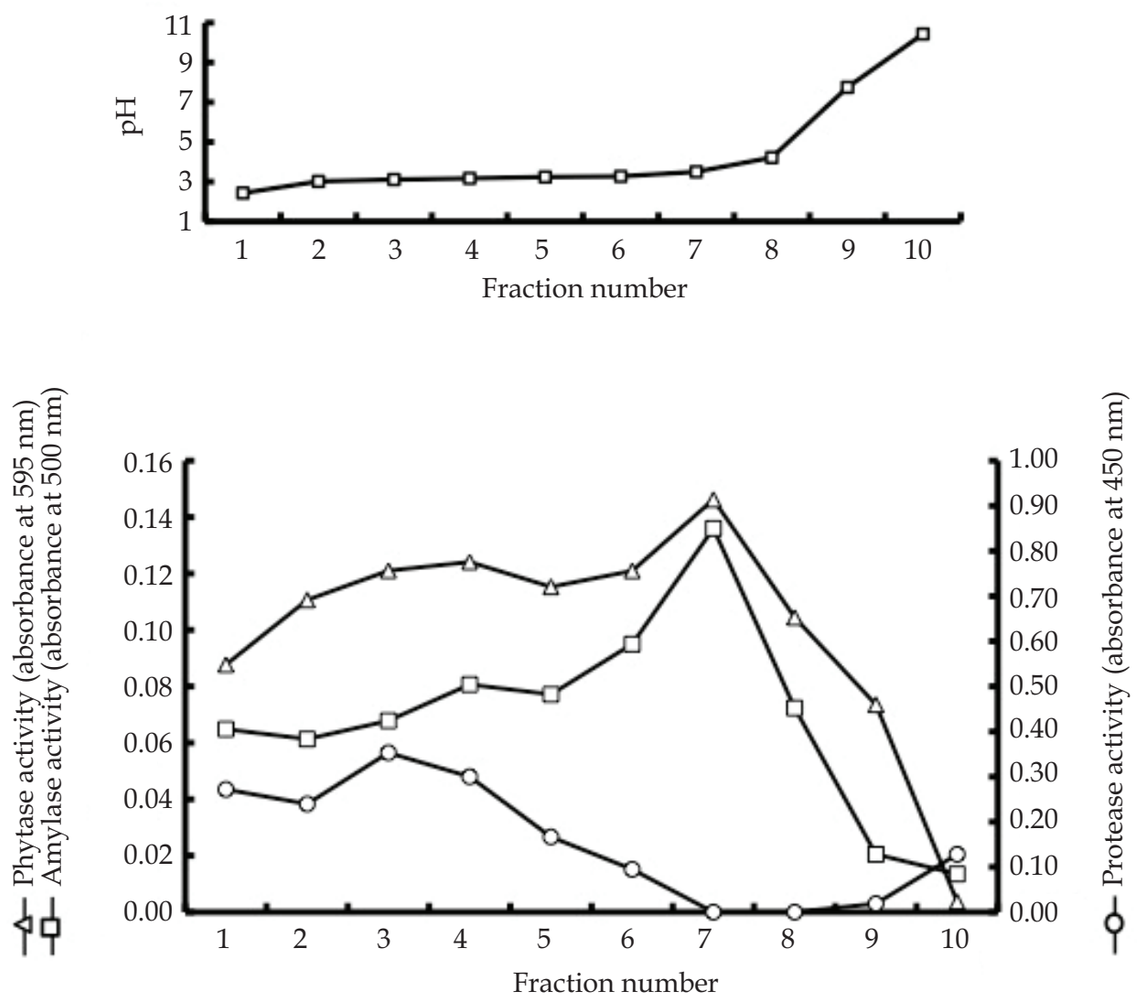



Fig. 8. Fractionation of phytase, amylase, and protease in a food additive-grade *Aspergillus niger* phytase preparation. pH gradient (upper) was formed by autofocusing for 24 hours. Fraction No. 7 and 9 were free from protease activity and used as high amylase and low amylase fractions, respectively

highest phytase and amylase activities. In the Fr. 9, the amylase activity dropped to 15% of the maximum activity, whereas relatively high level of the phytase activity (more than half of the activity in Fr. 7) was recovered in the Fr. 9. Then, Fr. 7 and 9 were collected and used as high amylase and low amylase fractions, respectively. These fractions were free from protease activity. The crude phyase preparation, which contains significant protease activity and two protease free autofocusing fractions were added to the 30% brown rice flour-added bread dough mix. Phytate content decreased by addition of all fractions. As shown in Figure 9, addition of the low amylase fraction did not significantly affect the loaf volume and did not induce collapse of crust up to 3000 U of phytase activity. Addition of the high amylase fraction showed positive effect on loaf volume up to 514 U of amylase activity. However, addition of amylase, more than 1029 U, decrease of loaf volume and partial collapse of crust (arrow) were observed. Addition of the crude preparation at 791 U of protease activity induced partial collapse of crust even amylase activity was less than 500 U. Addition of protease more than 1582 U resulted in extensive collapse of whole crust and deteriorated texture properties (mash-like texture).

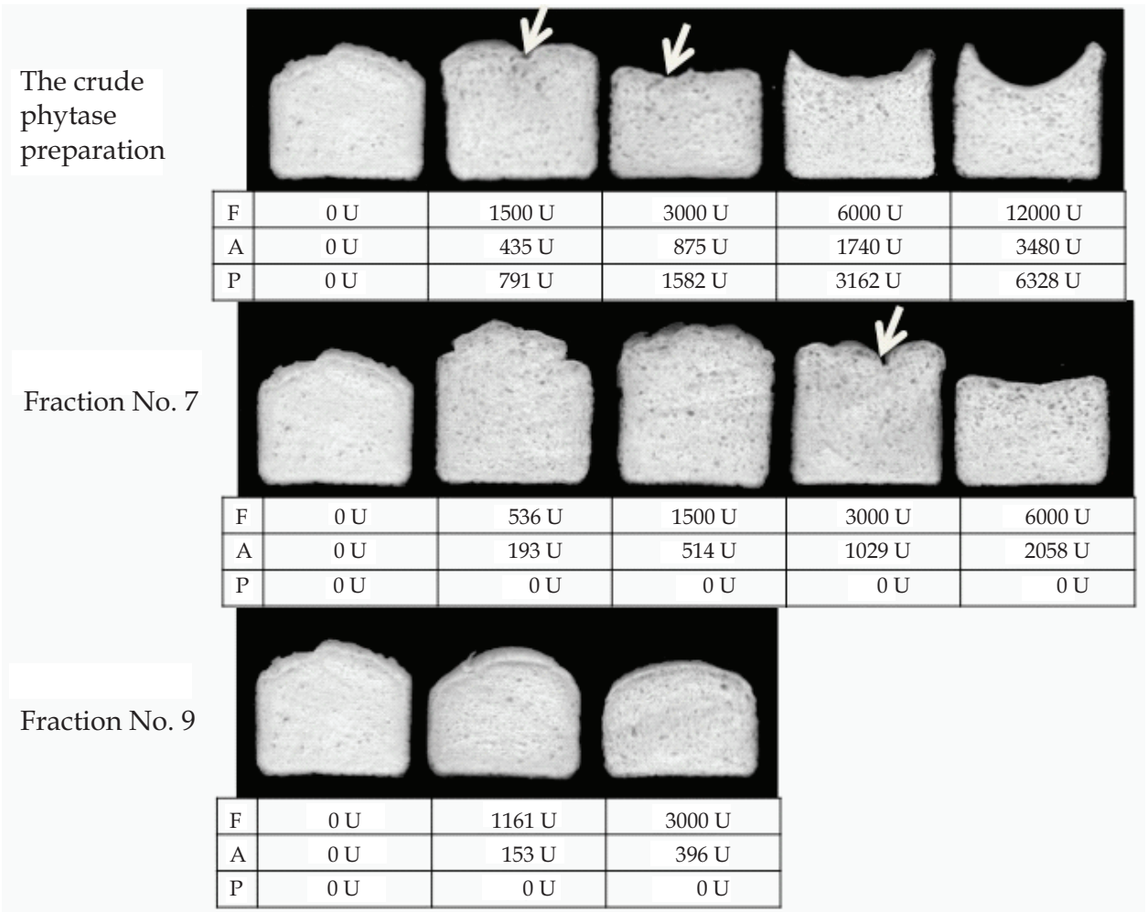



Fig. 9. Effects of addition of crude phytase preparation and high amylase (Fr. No. 7) and low amylase (Fr. No. 9) fractions on appearance and swelling properties of the 30% brown rice flour-containing bread. Phytase (F), amylase (A), and protease (P) activities are indicated in the tables

On the basis of these results, it can be concluded that $IP_6$ content in the unrefined grain flour-containing bread can be reduced by direct addition of the fungal phytase. However, presence of high protease activity in the phytase preparation can deteriorate swelling and textural properties and collapse the crust. On the other hand, amylase can improve the swelling property at relatively low activity (<500 U), while it deteriorates the swelling property at high activity (> 1000 U). These phenomena can be explained as follows. Protease in the preparation degrades gluten and deteriorates gluten network. Consequently, the dough can not retain the $CO_2$ gas generated by fermentation, which decreases loaf volume and finally collapses whole crust. The amylase in the preparation may induce oligosaccharides from starch and consequently enhance fermentation by yeast at the low amylase activity. On the other hand, the high amylase activity can deteriorate starch gel network after cooking process. Indeed, fungal amylase has been used in the baking industry to accelerate fermentation. However, addition of excessive amylase and protease has been demonstrated to make the dough slack (Fred et al., 1975). These facts clearly indicate that the control of protease and amylase activities is crucial for the application of the fungal phytase preparation to bread making to reduce $IP_6$ and obtain good quality of the product.

## 7. Conclusion and future prospects

It has been demonstrated that fungal phytase can be used to reduce $IP_6$ level of the soybean based products and also unrefined grain flour-containing products, especially bread. However, fungal phytase may contain protease and amylase, which may affect quality of the final products. In some cases, the product may be unacceptable due to excessive degradation of protein, which induces unfavorable effects on texture and appearance. Then it is crucial to control the contaminated enzyme activity in the phytase preparation. In the present chapter, ion exchange column chromatography and autofocusing can at least partially control contamination of protease and amylase activities. In regardless of the high resolution, column chromatography requires relatively expensive apparatus and high running cost. In addition, it may require salts, buffer, solvents etc, which should be removed from the final product. It also increases preparation cost. On the other hand, autofocusing does not require organic solvents and salts for fractionation and has inherited potential for scale-up. Recently, continuous type autofocusing apparatus has been developed (Hashimoto et al., 2006). Then this technique would be useful to control protease and amylase activities in the phytase preparation. By using this technique, a food additive phytase optimized for bread making and other applications could be produced.

$IP_6$ also suppresses divalent ions-induced Fenton's reaction; producing hydroxyl radical from hydrogen peroxide. Then $IP_6$ has been used to suppress oxidation of food (Graf and Eaton, 1990). However, little is known for the suppressive effect of $IP_1$-$IP_5$ against Fenton's reaction. If degradation products of $IP_6$ by phytase also suppress Fenton's reaction without reducing mineral bio-availability, phytase can be used for production of anti-oxidant food ingredient with excellent nutritional value. In addition, functional food based on suppression of Fenton's reaction without disturbing nutritional could be also produced by the limited digestion of $IP_6$ by phytase. For these purposes, further studies on control of hydrolysis of $IP_{2\text{-}6}$ by phytase are necessary.

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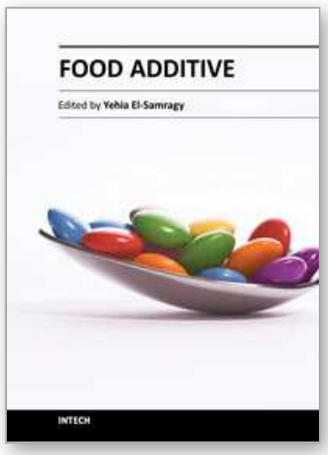
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**Food Additive**

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A food additive is defined as a substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food whether or not it has nutritive value. Food additives are natural or manufactured substances, which are added to food to restore colors lost during processing. They provide sweetness, prevent deterioration during storage and guard against food poisoning (preservatives). This book provides a review of traditional and non-traditional food preservation approaches and ingredients used as food additives. It also provides detailed knowledge for the evaluation of the agro-industrial wastes based on their great potential for the production of industrially relevant food additives. Furthermore the assessment of potential reproductive and developmental toxicity perspectives of some newly synthesized food additives on market has been covered. Finally, the identification of the areas relevant for future research has been pointed out indicating that there is more and more information needed to explore the possibility of the implementation of some other materials to be used as food additives.

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